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Ephrin-A1 Suppresses Th2 Cell Activation and Provides a Regulatory Link to Lung Epithelial Cells

Jan G. Wohlfahrt,* Christian Karagiannidis,* Steffen Kunzmann,* Michelle M. Epstein,† Werner Kempf,‡ Kurt Blaser,* and Carsten B. Schmidt-Weber2*

Gene expression screening showed decreased ephrin-A1 expression in CD4+ T cells of asthma patients. Ephrin-A1 is the ligand of the Eph receptor family of tyrosine kinases, forming the largest family of receptor tyrosine kinases. Their immune regulatory properties are largely unknown. This study demonstrates significantly reduced ephrin-A1 expression in T cells of asthma patients using real time-PCR. Immunohistochemical analyses revealed strong ephrin-A1 expression in lung tissue and low expression in cortical areas of lymph nodes. It is absent in T cell/B cell areas of the spleen. Colocalization of ephrin-A1 and its receptors was found only in the lung, but not in lymphoid tissues. In vitro activation of T cells reduced ephrin-A1 at mRNA and protein levels. T cell proliferation, activation-induced, and IL-2-dependent cell death were inhibited by cross-linking ephrin-A1, and not by engagement of Eph receptors. However, anti-EphA1 receptor slightly enhances Ag-specific and polyclonal proliferation of PBMC cultures. Furthermore, activation-induced CD25 up-regulation was diminished by ephrin-A1 engagement. Ephrin-A1 engagement reduced IL-2 expression by 82% and IL-4 reduced it by 69%; the IFN-γ expression remained unaffected. These results demonstrate that ephrin-A1 suppresses T cell activation and Th2 cytokine expression, while preventing activation-induced cell death. The reduced ephrin-A1 expression in asthma patients may reflect the increased frequency of activated T cells in peripheral blood. That the natural ligands of ephrin-A1 are most abundantly expressed in the lung may be relevant for Th2 cell regulation in asthma and Th2 cell generation by mucosal allergens. The Journal of Immunology, 2004, 172: 843–850.

DNA array-based screening of T cell cDNA of asthmatic patients revealed reduced ephrin-A1 expression compared with those of healthy donors (1). This finding is interesting because ephrin-A1 expression was previously not reported in peripheral CD4+ T cells and in addition Eph receptors are expressed on epithelial cells, which are important cells for the lung function. Therefore, ephrins may represent a regulatory interfacial epithelial surfaces in the lung and the immune system.

The ephrins are the ligands of the Eph receptors, which form with at least 14 members the largest group of tyrosine-kinase receptors (2–4). Ephrins are divided into two subclasses. The A subclass (ephrin-A1–A6) consists of proteins tethered to the cell membrane via a GPI anchor, whereas the B subclass (ephrin-B1–B3) consist of a transmembrane domain and a short cytoplasmic region (5, 6). Similarly, the Eph receptors are divided in an A (EphA1–9) and a B group (EphB1–6), dependent on the group of ephrins they are binding. All Eph receptors of the A subclass can bind ephrin-A1 (7), but the receptor EphA2 binds ephrin-A1 with the highest affinity (Kd = 25 nM (8)). All receptors of the EphA group can bind several ephrins beside EphA1, which can bind only ephrin-A1 (2, 7). The ligands of the Eph receptor family have to be membrane bound to be active. Therefore, cell-cell interaction is necessary for Eph receptor-ephrin signaling, and soluble ephrins are active only if they are artificially clustered (9–11).

Characteristic for the ephrin biology is that not only is a forward signaling pathway by the Eph receptors induced upon ligand binding but also the ligand itself transmits a reverse signals into the cell (4). Interestingly, even the GPI-anchored ephrin-A ligands can induce signals due to lipid raft microdomain-associated uncharacterized receptors, which are specifically phosphorylated after ephrin engagement (12–15). Involvement of Eph receptors and ephrins is currently discussed in developmental processes (16–19), as well as their role in carcinogenesis (20).

Human ephrin-A1 is a 25-kDa glycoprotein with high homology to murine and rat orthologs (21) and is abundantly expressed in lung, but is also found in kidney, salivary gland, skin, and intestine (21, 22). It was found as an immediate-early response gene of endothelium induced by TNF-α (23) that possesses angiogenic and endothelial cell chemoattractant activity (24). EphA1 receptor is the least conserved member of the Eph receptor family and is expressed in high levels in the liver, kidney, thymus, and lung but not in the brain (25, 26). Eph receptors are also transcribed at high levels in tumor cells of epithelial origin (27, 28) and overproduction of EphA1 induces tumorigenic ability of normal fibroblasts or melanoma progression (27, 29). This could support a role for ephrins in the growth of the cells, but it could also indicate that the immune system recognizes the ephrin-overexpressing cells to a reduced degree. In fact, recent studies showed that immuno-competent cells express Eph receptors and ephrin ligands. A soluble splice variant of ephrin-A4 is secreted by activated human B lymphocytes, and EphA2 receptors are present on dendritic cells/macrophages in tonsils (30) and peripheral blood (31). TCR engagement along with EphB6 receptor cross-linking in the Jurkat cell..

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line results in FAS-mediated cell death (32). EphB6 receptor over-exposure in this cell line suppresses TCR-mediated IL2 secretion and CD25 expression by inhibition of the c-jun kinase pathway (33). Furthermore, plate-bound ephrin-A1 inhibits strong chemotaxis in thymocytes and Jurkat cell line (34). These studies clearly demonstrate that ephrins participate in immune regulation and possibly convey an off signal to the immune system.

In this study, we describe ephrin-A1 expression by T cells and the impact of ephrin-A1 cross-linking on T cell function. The strong localized expression of Eph receptors on lung epithelia suggests that ephrins create microenvironmental conditions, which are known to contribute to the pathogenesis of asthma such as hyper-reactivity of the lung and airway remodeling.

**Materials and Methods**

**Patients**

All asthmatic patients met the American Thoracic Society definitions of asthma (35). At the time of the study, allergic asthma (AA) patients had stable, severe bronchial asthma; non atopic asthma; were not in acute respiratory distress; had no evidence of chest infection; and had never had atopic dermatitis (AD); but were all characterized by an atopic predisposition.

**Isolation of T cells**

PBMC were isolated from blood of nonsmoking healthy donors by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation. The interphase cells were washed three times, and CD4+ T cells were purified using anti-CD4 Dynal magnetic beads and Detach-a-Bead Abs (both Dynal, Hamburg, Germany) as previously described (36). The purity of CD4+ T cells was initially tested by flow cytometry and was >95%. CD19 B lymphocytes were isolated with CD19 MACS MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer’s protocol. The viability of the T cells was verified after isolation and was ≥99%.

**Cell cultures**

All cell cultures with human CD4+ T cells were grown in RPMI 1640 supplemented with 2 mM glutamine, 1 sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (all from Life Technologies, Basel, Switzerland) and 10% FBS (Sera-Lab, Loughborough, U.K.) in a humidified atmosphere containing 5% CO2 at 37°C.

**DNA-array hybridization**

CD4+ T cells were isolated out of 80 ml of peripheral blood from three patients with stable, severe bronchial asthma and three healthy persons. For RNA isolation, 5 × 10^6 purified CD4+ T cells were lysed with RNeasy lysis buffer (Qiagen, Hamburg, Germany) after overnight stimulation using plate-bound anti-CD3 (OKT3, 1 μg/ml) and anti-CD28 (15E8; Nethlands Red Cross blood transfusion service, Amsterdam, The Netherlands; 2 μg/ml). The OKT3 hybridoma was purchased from American Type Culture Collection (ATCC, Manassass, VA), and supernatants were purified using affinity chromatography. RNA was isolated using the RNEasy Mini Kit (Qiagen) and eluted in 100 μl of H2O. The RNA was precipitated with sodium acetate and ethanol, washed with 70% ethanol, and resuspended in 11 μl of H2O. The RNA was mixed with 4 μl of human array-specific primers (R&D Systems, Abingdon, U.K.) and incubated in a thermal cycler at 90°C for 2 min. The temperature was ramped to 42°C in 20 min. For reverse transcription, the following components were added: 6 μl of 5′ reverse first-strand buffer (Life Technologies, Basel, Switzerland); 2 μl of 0.1 M DTT (Life Technologies); 20 U RNase inhibitor (Roche, Mannheim, Germany); 1 μl of dCTP, dGTP, dTTP (each 10 mM; PerkinElmer), 5 μl of (α-32P)dATP (0.4 MBq/μl; Hartmann, Braunschweig, Germany); and 1 μl of Superscript II RNase H-reverse transcriptase (Life Technologies). The mixture was incubated at 42°C for 3 h for 3 h and diluted up to 100 μl with H2O. The cDNA was purified with the QiAquick Nucleotide Removal Kit (Qiagen) and eluted in 100 μl of H2O. An aliquot of 1 μl was measured in a scintillation counter to estimate the percentage incorporation of labeled nucleotides in the cDNA. CD4+ T cell cDNA probes were prepared from patients’ blood were hybridized in parallel with cDNA probes from healthy donors on human cytokine expression array membrane (R&D Systems) following the instructions of the manufacturer. After hybridization, membranes were washed for 20 min with 30 ml of a 0.1× standard saline-citrate-phosphate/EDTA, 1% (w/v) SDS solution. Subsequently, moistened arrays were placed in plastic bags and exposed for 24 h on phosphormager screens (Fuji Film, Tokyo, Japan). The screens were analyzed using the FLA 3000 phosphorimagery system (Fuji) and evaluated with the AIDA program (Raytest, Urdorf, Switzerland).

**T cell proliferation assay**

Triplicates of 1 × 10^5 CD4+ T cells or PBMC were set up in 200 μl of RPMI 1640 in 96-well flat-bottom microtiter plates (Costar, Corning, NY). Cells were activated by plate-bound mouse anti-human CD3 mAb (1 μg/ml; clone CRL 8001; ATCC) and mouse anti-human CD28 mAb (2 μg/ml; clone 15E8; Netherlands Red Cross blood transfusion service). The bottom of the wells were as well covered with rabbit Ig Fraction (DAKO-Cytomation, Zug, Switzerland), rabbit-anti-human ephrin-A1 (586, Biotechnology, Santa Cruz, CA), rabbit-anti-human EphA1 receptor (R&D Systems), or rabbit-anti-human EphA2 receptor Abs (Sigma-Aldrich, Buchs, Switzerland). The concentration of the plate-bound Abs was 4 or 1 μg/ml for soluble addition of Abs to the cultures. One triplicate of cells was not activated to calculate the stimulation index for the activated cells. After an incubation time of 1, 2, 3, or 6 days, cells were pulsed with 16 μl with 1 μCi [3H]thymidine (Hartmann) and harvested on glass fiber filters using an automated multisample harvester (Tomtec, Hamden, CT). Filters were transferred in sample bags with liquid scintillation fluid and analyzed using a β-scintillation counter (Pharmacia-Wallac, Turku, Finland).

**Quantitative real time PCR**

Total RNA was isolated from 3 × 10^6 CD4+ T lymphocytes using the RNeasy minikit (Qiagen) according to the manufacturer’s protocol. The RNA was separated in 50 μl of glycerol and 4 μl of total RNA (10 μl) were reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Roche/Applied Biosystems, Rotkreuz, Switzerland) with random hexamers as primer following the recommendations of the supplier in a total volume of 30 μl. The PCR primers and probes detecting ephrin-A1, EphA1 receptor, and IL-2 were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Perkin Elmer/Applied Biosystems, Foster City, CA) as follows: ephrin-A1 forward primer 5′-CTA TAC ATG TAC GCC TGA AC-3′; ephrin-A1 reverse primer 5′-CAG CGT CCG CCA CAG AGT GA-3′; ephrin-A1 probe FAM 5′-ACG TGG ACA TCA CAG AGT GA-3′, TAMRA; ephA1 forward primer 5′-TTG TGG AGT CCC AAG AAG-3′; ephA1 reverse primer 5′-TGAG ATC CCT GGT ACA TG-3′; EphA1 probe FAM 5′-AGT GTG GTG TGA ACA GCA ACA GAT AC-3′; TAMRA; IL-2 forward primer 5′-TCA CCA GGA TGC TCA ATG ATG GA-3′ and 3′-CTT CAG GGT TTA GAG TGG TTA GGT CT-3′; IL-2 reverse primer 5′-GAG GTG TTA CCT CCT CTT ACT ACT AC-3′; IL-2 probe FAM 5′-ATG CCC AAG AAG GGC ACA GAA CGT AAA C-3′; TAMRA. For EphA2, IL-4, β-glucuronidase (GUS), and 18S rRNA, commercially available primers and probes were purchased (Applied Biosystems). The TaqMan probes of the target genes were labeled with FAM as the reporter dye and TAMRA as the fluorescence quencher. The probes for the housekeeping genes were labeled with VIC (Applied Biosystems) as the reporter dye and TAMRA as fluorescent quencher. When possible, primers were spanning exon-intron borders to eliminate amplification of contaminations of genomic DNA. Accumulation of the PCR products was detected in real time by monitoring the probe cleavage-induced mobilization of the reporter dye. The prepared cDNAs were amplified using an UNG-containing PCR mastermix (Perkin Elmer/Applied Biosystems) according to the recommendations of the manufacturer in a total volume of 25 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). To determine a correct PCR efficiency, cDNA samples were amplified with the same forward and reverse primers. Amplification was performed using the comparative ΔΔCt method as described (37, 38). All amplifications were conducted at least in duplicates.

**Immunoblotting**

CD4+ T cells or CD19+ B cells 2 × 10^6 were lysed in Laemmli buffer (125 mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 2.5% 2-ME, bromphenol blue) and homogenized with a Quashredder (Qiagen). Lysates were heated for 10 min to 75°C and loaded next to a Benchmark protein-mass ladder.

3 Abbreviations used in this paper: AA, allergic asthma; GUS, β-glucuronidase; TBS, Tris-buffered saline; AICD, activation-induced cell death.
(Invitrogen, Basel, Switzerland) on a NuPAGE 4–12% bis-tris gel (Invitrogen) and run until proteins were sufficiently separated. The proteins were electroblotted onto a nitrocellulose membrane (Amersham Life Science, Dübendorf, Switzerland). The membrane was blocked overnight with TBS containing 5% BSA fraction V (Sigma-Aldrich), washed three times for 5 min, and incubated with 1 μg/ml rabbit anti-human ephrin-A1 Ab (Santa Cruz Biotechnology) in TBS buffer with 5% BSA fraction V for 1 h. The blot was washed three times with TBS after incubation with HRP-labeled donkey anti-rabbit Ig (Amersham Life Science) in a dilution of 1/50,000 in TBS containing 5% BSA fraction V for 1 h. The blot was developed using the ECL Plus System (Amersham Life Science) following the recommendation of the supplier.

**Flow cytometry**

After stimulation with plate-bound anti-CD3 Ab and anti-CD28 Ab for 1 and 16 h, 1 x 10^6 unstimulated and stimulated CD4^+ T cells were incubated for 45 min with 1 μg of a rabbit anti-human ephrin-A1 Ab (Santa Cruz Biotechnology). After a washing, the first Ab was stained with 5 μg of a FITC-conjugated F(ab')2 fragment of a swine anti-rabbit Ab (Dianova, Hamburg, Germany). Cell were fixed with 2% paraformaldehyde and subjected to FACS (EPICS XL-MCL, Beckman Coulter, Miami, FL). CD25 expression was measured accordingly, using an RD1-labeled anti-CD25 Ab (Beckman Coulter). Viabilities of cell populations were measured by FACS using 25 μM ethidium bromide.

**Allergic asthma induction in mice**

BALB/c mice were immunized i.p. with 10 μg of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in 200 μl of PBS or with PBS alone and on days 0 and 21, and 1 wk later on days 28 and 29 they were nebulized with 1% OVA in PBS using an ultrasonic nebulizer for 60 min twice daily, as previously described (39). Mice were then rechallenged a second time with aerosolized 1% OVA or PBS for 60 min twice daily on 2 consecutive days on days 89 and 90. After 48 h, lungs were perfused with PBS and fixed with 4% formalin. Memory, recovered, and control groups are referred to as OVA-OVA-OVA (OVA_i.p. -OVA aerosol -OVA aerosol), OVA-PBS-OVA (OVA_i.p. -PBS aerosol -OVA aerosol), and PBS-PBS-PBS (PBS_i.p. -PBS aerosol -PBS aerosol).

**Immunohistochemical staining**

Paraformaldehyde-fixed sections were incubated overnight at 55°C. Sections were deparaffinized by 2 x 5 min of incubation in xylene followed by 2 x 3 min of incubation in 100, 95, 90, 80, and 70% ethanol. Slides were heated for 5 min to 90°C in 10 mM sodium citrate buffer (pH 6.0). Cold slides were incubated in peroxidase block buffer of the DAKO EnVision + System Peroxidase (3-amino-9-ethylcarbazole) for mice (DAKOCytomation) for 5 min and washed with TBST (0.05 M Tris, 0.3 M NaCl, 0.1 M Tween 20, pH 7.4). Slides were pre-incubated with 25% FCS, 0.1% BSA in TBST for 20 min and washed: anti-ephrin-A1, anti-EphA1, and anti-EphA2 (Santa Cruz Biotechnology) or rabbit Ig fraction (DAKOCytomation) as isotype control were added at a concentration of 1 μg/ml in blocking buffer for an incubation period of 16 h at 4°C. Biotinylated rabbit anti-mouse IgG and HRP-conjugated streptavidin (DAKOCytomation) was added for 30 min at room temperature. After washes, Anti-ephrin-A1 staining of (A) and EphA1 receptor expression by epithelial cells in bronchi. The black arrow in (D) highlights epithelial cell thickening, and the blue arrow perivascular, peribronchial infiltrates. Values are representative of at least three independent experiments.
used for detection, and AEC–substrate (DAKOCytomation) was used as substrate as described by the manufacturer. All sections were counterstained with hematoxylin and analyzed using an Axiovert microscope and an AxioCam digital camera (both Zeiss, Göttingen, Germany).

**Statistical analysis**

The data were tested for significance using the Mann-Whitney U test. Differences of \( p \leq 0.05 \) were considered to be significant.

**Results**

**Expression of ephrin-A1 in T cells**

Screening for gene expression changes of 475 different genes in cDNA libraries of activated CD4\(^+\) T cells of three AA patients compared with three healthy persons using the cDNA array technology showed a decreased expression of the mRNA of ephrin-A1. The intensity of the array spots were 1.9-fold lower (\( \leq 0.28 \)) in the AA patients than in the healthy counterparts (Fig. 1A). We confirmed this result using the quantitative real time RT-PCR method. Comparing the mRNA levels of peripheral CD4\(^+\) T cells of five other patients with severe AA and of five healthy volunteers, again the ephrin-A1 level in the cells of the AA patients was 2.0-fold (\( \leq 0.65; \ p \leq 0.05 \)) decreased (Fig. 1B). To show the expression of ephrin-A1 in CD4\(^+\) T cells on the protein level, lysates of CD4\(^+\) T cells and CD19\(^+\) B cells were immunoblotted. In both cases, a single band at 21 kDa was visible. Ephrin-A1 is expressed in higher amounts in CD4\(^+\) T lymphocytes than in CD19\(^+\) B lymphocytes (Fig. 1C). The EphA1 and EphA2 receptors were expressed only in background levels as mRNA and as protein (data not shown).

**Ephrin-A1 and Eph-A1 expression in vivo**

Ephrin-A1 expression was analyzed in lymphoid organs of NMRI mice. Ephrin-A1 expression was low in spleen (Fig. 2A) and was predominantly observed in the marginal zone areas around the lymphoid follicles. In lymph nodes, ephrin-A1 was detected predominantly in cortical areas. The EphA2 receptor was expressed at low levels in the red pulp of the spleen (data not shown) but was totally absent in the T or B cell-rich white pulp of the spleen. Neither EphA2 nor EphA1 receptors were visible in inguinal and
mesenteric lymph nodes, whereas ephrin-A1 expression was low and predominantly in cortical areas of the lymph nodes (data not shown). In contrast, ephrin-A1 expression was frequently observed in bronchi (Fig. 2B). Furthermore, strong EphA1 receptor expression was observed on any epithelial cell of small and large bronchi. This expression pattern was also observed in OVA-sensitized mice 90 days after OVA aerosol challenge. In inflammatory infiltrates can be observed, containing scattered and weak EphA1 receptor-expressing cells. Epithelial cells appear thickened and slightly lower in EphA1 receptor expression (Fig. 2D). Similarly, EphA1 receptor and ephrin-A1 expression was found on epithelial cells of human asthmatic lung sections. Ephrin-A1 was also found on scattered subepithelial cells.

**Regulation of ephrin-A1 in T cells**

Because DNA array comparison showed a decreased ephrin-A1 expression in cell populations of CD4\(^+\) T cells of the peripheral blood of AA patients compared with those of healthy persons, we asked whether observed changes are due to a disease-mediated activation of T cells and whether the level of ephrin-A1 expression is changed after engagement of the TCR. The expression of ephrin-A1 is reduced in CD4\(^+\) T cells after activation on both the mRNA and the protein level. Quantitative real time RT-PCR of isolated mRNA reveals that ephrin-A1 expression of CD4\(^+\) T cells stimulated for 1 h was 2.0-fold (± 0.6) and 3.7-fold (± 0.8) reduced after 16 h of stimulation with plate-immobilized anti-CD3 and anti-CD28 mAb (Fig. 3, A and B). The real time-based quantifications were performed in the linear range of ephrin-A1, and all other genes were quantified in the present study (data not shown). Flow cytometric analysis clearly demonstrates ephrin-A1 expression on 9.52 ± 0.12% CD4\(^+\) T cells (Fig. 3C). However, after stimulation of T cells, ephrin-A1 disappears from the cell surface within 1 h and remained absent (16 h; Fig. 3C).

**Ephrin-mediated inhibition of T cell proliferation**

The expression results demonstrated that ephrin-A1 is activation dependently expressed and thus may also be itself functionally involved in the regulation of the cellular activation process. Proliferation assays of activated CD4\(^+\) T cells demonstrate the role of the expression of ephrin ligands in the regulation of lymphocytes. Plate-bound anti-ephrin-A1 Ab provoked a significant decrease of anti-CD3/28-induced T cell proliferation 3 days after T cell activation. The proliferation index was significantly lower (p ≤ 0.001; 33.5 ± 9.6-fold) compared with T cells stimulated in the presence of the isotype control. In contrast, anti-EphA1 receptor Ab and anti-EphA2 receptor Ab (Fig. 4A) induced only a minor decrease.

**FIGURE 4.** A. \[^{3}H\]Thymidine incorporation of CD4\(^+\) T cells after 3-day culture on plate-immobilized anti-CD3 and CD28 along with Abs as indicated. The stimulation index was calculated by the division with the respective medium control. Bars, SEM of six independent experiments; *, statistical significance (p ≤ 0.001). B. T cells were stimulated on plate-immobilized anti-CD3 and CD28 along with Abs as indicated and rested for 10 days. The restimulation was performed as with anti-CD3/CD28 without any anti-ephrin Abs; ■, mean of three independent experiments (n = 3). C, CD25 (IL-2R\(\alpha\)) surface expression of anti-ephrin-A1-treated cells. Cells were treated as described for A. Gray histogram, CD25 staining; clear histogram, isotype control. The histograms are representative of three independent experiments.
The reduced proliferation was visible at days 5 and 7 after stimulation (data not shown). Anti-ephrin-A1 inhibited similarly effective the PHA-stimulated proliferation of T cells as TCR-specific stimulation (data not shown). Proliferation assays performed with CD4/H11001 T cells isolated from asthmatic donors were not different from those of healthy donors. The inhibition of proliferation was dose dependent in a range of 0.04 to 40/H9262 g (data not shown). Restimulated, anti-ephrin-A1-treated cells did not show anergy (Fig. 4B). Anti-ephrin-A1 treatment of the cells did not affect the viability of the unactivated T cells and was always >87.2 ± 0.3% after 3 days (Fig. 6A) and 84.8 ± 0.9% after 5 days (data not shown), as tested by ethidium bromide exclusion. The dramatic effect of anti-ephrin-A1 also effectively reduced (7.3 ± 2.3-fold) the surface expression of the early activation marker CD25 (Fig. 4C).

Incubation with anti-EphA1 receptor Abs

To verify that not only Ab-mediated cross-linking of ephrin-A1 affects T cell proliferation, but also the natural ligand-receptor interaction, we blocked the EphA1 receptor with Abs in cultures containing APC. To separate effects of anti-EphA1 on T cells vs APC, we pretreated irradiated CD4/H11002 PBMC or CD4/H11001 T cells with anti-EphA1 receptor Abs. Although the intensity and/or frequency of EphA1 is very low (31), we observed a slightly, but consistently enhanced proliferation in Ag-specific (Fig. 5A) and polyclonal T cell responses (Fig. 5B). The effect on the Ag-specific response was observed only among anti-EphA1 receptor APC, whereas anti-EphA1-pretreated T cells proliferated normally.

Incubation with anti-ephrin-A1 Abs reduces activation-induced cell death (AICD) of activated CD4/H11001 T cells

The pronounced effect on IL-2 cytokine and receptor expression is likely to affect AICD, which is known to be IL-2 dependent (40). We therefore induced AICD with plate immobilized anti-CD3 mAb in the presence and absence of anti-ephrin-A1 Abs. In fact, anti-ephrin-A1 prohibited AICD to large extent, whereas it did not induce cell death in resting T cells (Fig. 6A). However, addition of exogenous IL-2 rendered ephrin-A1 engagement ineffective to induce AICD (Fig. 6B).

Ephrin-A1 engagement reduces the expression of IL-2 and IL-4 but not IFN-γ in activated T cells

Because an increased production of IL-2 is typical for activated T cells, we analyzed the effect of an incubation of anti-ephrin-A1 Ab on the expression of IL-2 mRNA of activated CD4/H11001 T cells using the quantitative real time RT-PCR method. After 3 days, the incubation of activated CD4/H11001 T cells with anti-ephrin-A1 Ab led to a 21.3 ± 2.4-fold decrease of IL-2 mRNA (Fig. 7, A and B) and a 12.9 ± 3.2-fold decrease of IL-4 (Fig. 7, C and D) compared with activated T cells without this Ab. In contrast, IFN-γ expression was not affected (± 0.2; data not shown). The results were confirmed by ELISA; however, total amounts of IL-4 were very low.

Discussion

Activated CD4/H11001 T cells from asthma patients have decreased expression of ephrin cDNA compared with healthy controls (41, 42). The present findings confirm reduced ephrin-A1 expression in patients suffering from severe asthma and verifies the presence of ephrin-A1 protein in T cells and CD19/H11001 B cells (30). Most studies
to date support the notion that ephrin-mediated signals are suppressive. Thus, we speculate that diminished ephrin-A1 expression in allergic disease and an apparent suppressive effect of ephrins may be associated with a lack of suppression and, in turn, a loss of peripheral tolerance, as is often observed in allergic diseases.

We observed that ephrin-A1 was marginally expressed in lymph nodes and spleen and that expression of the receptors was not in T cell-rich areas (e.g., white pulp), confirming previous studies demonstrating that EphA2 receptor is expressed only in dendritic cells in the crypts of human tonsils (30). In addition to peripheral lymphoid expression, overlapping expression of ephrin-A1 and EphA1 receptors has been shown in rat thymus (43). Remarkably, we observed that EphA1 receptors were strongly and homogeneously expressed by large and small bronchi in mouse and human biopsy samples. In a mouse model of OVA-induced allergic lung inflammation, we observed epithelial thickening and a slight decrease in EphA1 receptor expression in areas in close proximity to inflammatory infiltrates. Taken together, these data demonstrate high expression in thymus and bronchial-associated lymphoid tissue, which are both epithelial-rich tissues. High expression of EphA1 receptor within the lungs and the changes in ephrin-A1 expression during allergic disease may not be merely a consequence of asthma but may relate to disease pathogenesis.

Activation of T cells by plate-immobilized anti-CD3 and anti-CD28 mAb induced a strong, immediate, and long-lasting (50–80%) decrease in ephrin-A1 expression compared with resting T cells both at the RNA and protein level, suggesting that reduced ephrin-A1 expression in asthma patients is likely due to recent T cell activation. This is supported by the observations that peripheral T cells of asthma patients have a high frequency of cells bearing the activation marker CD25 (44, 45), CD69 (46), or HLA-DR (44). Anti-EphA1 receptor or anti-EphA2 receptor marginally reduced proliferation, whereas anti-ephrin-A1 Abs coimmobilized with anti-CD3/28 mAb significantly diminished clonal expansion. Consistently, anti-EphA1 receptor Abs enhanced slightly Ag specifically and polyclonally induced T cell proliferation, despite the fact that EphA1 receptor is expressed only at very low levels on APC of peripheral blood (31). These data show that T cells can be inhibited by ephrin A1 and EphA1 receptor interactions.

The fact that T cells of healthy and asthmatic patients were equally suppressed, was expected, because low ephrin-A1-expressing T cells of asthmatics are preactivated in vivo and thus cannot by further activated in vitro and in turn show proliferative responses comparable with those of healthy donors. The decrease in T cell proliferation correlated with anti-ephrin-A1-mediated suppression of the IL-2Rα chain (CD25). These results support previous studies showing that EphB6 cross-linking prevents anti-CD3-induced CD25 up-regulation in Jurkat cells (33) and suggests that ephrins may influence cytokine-mediated immune regulation. The dramatic effect of ligand cross-linking also confirms the bidirectional nature of ephrin signaling, in which GPI-linked ephrins can specifically induce signal transduction by interacting with signaling competent receptors in lipid rafts (4).

The reduction in T cell proliferation was not due to T cell anergy, because anti-ephrin-A1-treated cells could be restimulated, and was independent of the induction cell death. In contrast, ephrin-A1 engagement prevented IL-2-dependent AICD, which depends on IL-2-dependent expression of FAS ligand (40). Ephrin-A1-mediated AICD prevention was reversed by addition of exogenous IL-2, which strongly suggests that ephrin-A1 directly affects the IL-2 pathway and is likely to underlie the observed difference in proliferation assays. Moreover, the absence of IL-2 receptor (CD25) expression upon anti-ephrin treatment implies an ephrin-mediated effect on IL-2, further
supported by a strong reduction of IL-2 mRNA expression (82%) when T cells were stimulated with anti-ephrin-A1 Abs, co-immobilized with anti-CD3/CD28 mAb. Additionally, incubation with anti-ephrin-A1 reduced IL-4 mRNA (69%) but had no effect on IFN-γ production. These findings are particularly important for allergic diseases, which are dominated by a Th2-like cytokine pattern. Taken together, these data suggest ephrin-mediated cytokine regulation, and it is tempting to speculate that in allergic disease, ephrin receptor-ligand interactions perturb peripheral suppression, thus leading to enhanced Th2 cell expansion.

The present study demonstrates for the first time ephrin-A1 expression in T cells and lung. The abundant presence of ephrin-A1 and its main receptors EphA1 and EphA2 in the lung suggests a regulatory interface of the immune system and the physiologically important epithelial surface in the lung. The impacts of ephrin-A1 and EphA receptors are of a suppressive consequence for the recipient T cell. It appears that T cells, which infiltrate bronchial tissues are hindered in IL-2 and IL-4 expression, whereas AICD is supposed to be decreased in a ephrin/Eph receptor-dominated microenvironment, provided that experimental, Ab-mediated ephrin-A1 cross-linking reflects the physiological receptor ligand interaction. The physiological relevance of such an immunoprivilege may be seen in the important physiological function of the lung. Because ephrin signaling is bidirectional, it can be expected that T cell-mediated ephrin contact also affects the function of the bronchial epithelial cells. These effects are the subject of current investigations in our laboratory. The surface-exposed expression of the ephrin system along with its negative regulatory function for the immune system, and possibly also for the epithelial cells, identifies these genes as pharmacological targets and important molecules for the pathogenesis of asthma.

References